Phenotypic and DNA Relatedness between Nematode Symbionts and Clinical Strains of the Genus *Photorhabdus* (Enterobacteriaceae)[†]

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Bacterial strains isolated from wide ranges of nematode hosts and geographic sources and strains isolated from human clinical specimens were used to assess the taxonomic structure of the genus *Photorhabdus*. The following two methods were used: DNA relatedness and phenotypic characterization. Analysis of the DNA relatedness data revealed that all of the strains studied were congeneric and that the genus *Photorhabdus* is, on the basis of DNA relatedness data, more homogeneous than the other genus of nematode-symbiotic bacteria, the genus *Xenorhabdus*. In contrast to previous reports, only two DNA relatedness groups were identified in the genus *Photorhabdus*. These groups corresponded to the symbiotic strains and the clinical strains. There appeared to be some subgroups within the symbiotic strain group on the basis of the interactions of the strains with nematodes, which corresponded to some extent with the DNA relatedness data. However, there were significant ambiguities in the DNA relatedness data, and this group could not be subdivided on the basis of DNA relatedness data or phenotypic data. The distinct functional differences within and between the DNA relatedness groups of symbiotic *Photorhabdus* strains indicated that there are biologically significant subgroups within the genus *Photorhabdus* that cannot be defined at this time. Further investigation of the taxonomy of *Photorhabdus* by using different approaches and a suitably wide range of strains is recommended. However, it is clear that the clinical strains form a recognizable subgroup within the genus even though no formal subtaxon can be defined at this time.

Khan and Brooks (28) and Poinar et al. (34) reported the isolation of bioluminescent bacteria that were symbiotically associated with the insect-pathogenic nematodes *Heterorhabditis bacteriophora* (synonym, *Heterorhabditis heliothidis* [30]) and *H. bacteriophora*, respectively. These bacteria were subsequently classified in the genus *Xenorhabdus* along with the symbionts of nematodes of the family Steinernematidae and described as members of a new species, *Xenorhabdus luminescens* (40). Bacteria subsequently isolated from *Heterorhabditis* spp. (3, 6, 7, 32, 33, 38) and some isolates obtained from clinical sources (20) were also classified as *X. luminescens* strains. On the basis of phenotypic characteristics and DNA relatedness data, the creation of a new genus, the genus *Photorhabdus*, to accommodate the bacteria previously identified as *X. luminescens* was proposed by Boemare et al. (14); only one *Photorhabdus* species, *Photorhabdus luminescens*, was recognized.

The results of several studies have indicated that *P. luminescens* should be divided into several groups. Initially, four DNA relatedness groups were recognized (24) in the genus *Photorhabdus*, and later a fifth group was recognized (20). Significant dissimilarities between isolates of *P. luminescens* have also been revealed by 16S rRNA studies (35). In addition, there are substantial differences in the interactions between *P. luminescens* and *Heterorhabditis* spp. in monoxenic cultures; the symbionts of some nematode isolates are not able to support cultures of other isolates (8, 22, 25). However, despite the

obvious differences between some *Photorhabdus* strains, the numbers of isolates used in the previous studies have been insufficient to permit subdivision of the genus.

One of the difficulties in establishing a taxonomy for these bacteria arises from phase variation. Akhurst (1a) showed that the symbionts of *Steinernema* and *Heterorhabditis* species occur in two phases that differ in their ability to support reproduction of the nematodes. The two phases of each *Xenorhabdus* and *Photorhabdus* species have been shown to vary in many characters (dye adsorption, antibiotic production, phospholipase activity, inclusion proteins, fimbriae, specific activities of respiratory enzymes) (8, 37); the two phases of *Photorhabdus* strains also differ in bioluminescence and pigmentation (6, 7, 12). The differences between the two phases are so marked that a numerical taxonomic study revealed that the phase characters strongly distorted the analysis and had to be discarded.

In this study we addressed the problems posed to previous workers by the limited range of strains tested and the differences between phases by using both phases of *Photorhabdus* strains isolated from wide ranges of nematode host species and geographical sources, including some clinical specimens, to test the hypothesis that the clinical strains form a subtaxon within the genus *Photorhabdus*.

MATERIALS AND METHODS

Bacterial isolates. Bacteria were obtained from broad ranges of nematode host species and geographical sources and from clinical specimens. The sources of the bacterial isolates used are listed in Table 1. Usually, phase I isolates were obtained directly from the nematode host (7); the strain Hb^T (T = type strain) culture was established from a single colony of strain ATCC 29999^T, which, like ATCC 29304, was obtained from the American Type Culture Collection, Rockville, Md. Phase II isolates were obtained from in vitro cultures of phase I isolates by repeated clonal selection of flat, nongranulated, nonmucoid colonies from stationary-phase nutrient broth cultures plated onto nutrient agar. Clones were considered phase II isolates only if, after serial subculture, they exhibited no

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[†] Dedicated to Lucienne Baud (deceased 9 August 1994) in memory of her contribution.

TABLE 1. Sources of *P. luminescens* strains used in this study

Strain	Other designation(s)	Geographical source	Nematode host species	Reference(s)
ATCC 29304	NC-19	United States	<i>Heterorhabditis bacteriophora</i>	20, 24, 28
C1	C, NC1	United States	<i>Heterorhabditis bacteriophora</i> ^a	3, 4
C8404		People's Republic of China	<i>Heterorhabditis</i> sp. ^b	4
C8406		People's Republic of China	<i>Heterorhabditis bacteriophora</i> ^c	4
D1	D	Australia	<i>Heterorhabditis</i> sp. ^b	3
Habana		Cuba	<i>Heterorhabditis</i> sp.	9
Hb ^{Td}	ATCC 29999 ^T , Hb1 ^T	Australia	<i>Heterorhabditis bacteriophora</i> ^c	34
Hm		United States	<i>Heterorhabditis bacteriophora</i>	12
HI		Italy	<i>Heterorhabditis</i> sp.	7
HF85		The Netherlands	<i>Heterorhabditis megidis</i>	38
HL81		The Netherlands	<i>Heterorhabditis megidis</i>	38
HFR86		The Netherlands	<i>Heterorhabditis megidis</i>	40a
HP88		United States	<i>Heterorhabditis</i> sp. ^a	32
HV16	V16	Australia	<i>Heterorhabditis</i> sp. ^a	4
HW79		The Netherlands	<i>Heterorhabditis megidis</i>	38
ItH211		Italy	<i>Heterorhabditis</i> sp.	16
K80		Argentina	<i>Heterorhabditis</i> sp.	18
K81		Guadeloupe	<i>Heterorhabditis</i> sp.	15
Meg		United States	<i>Heterorhabditis megidis</i>	33
NC162		United States	<i>Heterorhabditis bacteriophora</i>	4
NZH		New Zealand	<i>Heterorhabditis zealandica</i>	4, 41
Q614	HQ614	Australia	<i>Heterorhabditis</i> sp. ^c	4-6
T327		Australia	<i>Heterorhabditis zealandica</i>	4
Tetuan		Cuba	<i>Heterorhabditis</i> sp. ^f	4, 10
X1Lit		Lithuania	<i>Heterorhabditis</i> sp. ^g	4
X1Nach		Russia	<i>Heterorhabditis</i> sp. ^g	4
1216-79	ATCC 43948	United States	None	20
2407-88	ATCC 43952	United States	None	20
2617-87	ATCC 43951	United States	None	20
3105-77	ATCC 43949	United States	None	20
3265-86	ATCC 43950	United States	None	20

^a The results of cross-breeding and satellite DNA analyses indicate that *Heterorhabditis* sp. strain HP88 is not a *Heterorhabditis bacteriophora*, *Heterorhabditis indicus*, or *Heterorhabditis megidis* strain (1, 17). Joyce et al. (27) have suggested that this strain belongs to the same subgroup of the *Heterorhabditis bacteriophora* species complex as strains NC1 and HV16.

^b *Heterorhabditis* sp. strains D1 and C8404 were assigned to the same (undescribed) species on the basis of isozyme data (4).

^c An isozyme analysis of strains Hb and C8406 revealed that they are identical for 10 discriminating enzymes (4).

^d Hb^T was clonally derived from ATCC 29999^T.

^e Poinar (30) considered Q614 conspecific with NZH and T327 and to be a *Heterorhabditis zealandica* strain. However, see the compatibility data in Table 4.

^f Arteaga-Hernandez and Mracek (10) found no morphological differences between the Tetuan and P₂M strains of *Heterorhabditis* sp., and Joyce et al. (27) identified P₂M as a *Heterorhabditis indicus* strain on the basis of cross-breeding data and its restriction fragment length polymorphism profile. However, morphological characterization of *Heterorhabditis* strains has often been misleading, and so identification of the Tetuan strain as a *Heterorhabditis indicus* strain cannot be made with certainty.

^g Isozyme analysis of *Heterorhabditis* sp. strains X1Nach and X1Lit revealed that these strains are identical for 10 discriminating enzymes (4). Poinar (30) considered these strains conspecific with NZH, Q614, and T327 and to be *Heterorhabditis zealandica* strains.

evidence of antibiotic production or dye adsorption from MacConkey agar and did not regenerate phase I or intermediate variants (8, 22, 26).

Five isolates (1216-79, 2407-88, 2617-87, 3105-77, and 3265-86) were not associated with nematodes but were obtained from clinical sources (20); these isolates, which were provided by J. J. Farmer, Jr. (Centers for Disease Control and Prevention, Atlanta, Ga.), were identified as phase II isolates by their colony and cell morphology and by their lack of antibiotic production and dye adsorption. Phase I isolates of these strains have never been reported and could not be detected in this study. Except for the clinical isolates, the suffixes of the designations indicate phases (e.g., isolates Hb/1 and Hb/2 are phase I and phase II variants of isolate Hb^T, respectively), as previously described (13).

The DNA relatedness analysis component of the study was conducted in Australia, and the phenotypic analysis component was performed in France. At the beginning of the study, subcultures of each isolate were exchanged to ensure that the French and Australian groups worked with the same material. Reference cultures were stored in 17% (vol/vol) glycerol broth at -70°C. Stock cultures were maintained and monitored for phase variation as described by Boemare and Akhurst (13).

DNA relatedness. The methods used to purify bacterial DNA, hybridize DNA, and estimate DNA relatedness were those used by Boemare et al. (14). For 21 of 30 isolates DNA was purified only from phase I cultures. For isolates Hb^T, C1, Meg, K81, and T327, DNA was purified from both phase I and phase II cultures. As the clinical isolates did not occur as phase I organisms, DNA was purified from phase II cultures of strains 1216-79, 2617-87, 3105-77, and 3265-86.

DNA labelling with ³²P, reassociating DNA by a hydroxyapatite method at 60°C, and estimating DNA relatedness were carried out as described by Boemare et al. (14). Hybridization of phase I and phase II DNAs was performed with

isolates Hb^T, Meg, and K81. Reciprocal hybridizations (i.e., hybridizations in which first one isolate and then the other was used as the labelled isolate) were performed with 25 pairs of isolates. Replicate hybridization experiments were performed with many but not all pairs. For the pairs for which replicate hybridization experiments were performed a one-way analysis of variance was used to determine the significance of differences.

Phenotypic characterization. Most tests were conducted by using API 20E, API 20NE, API 50CH, API 50AO, and API 50AA strips (Biomérieux) as described previously (13). Tests for catalase activity, bioluminescence, dye adsorption from MacConkey agar, and phospholipase (lecithinase) activity were conducted by the methods employed by Akhurst (3). The methods of Boemare and Akhurst (13) were used to test for production of phenylpyruvic acid from phenylalanine, production of indolepyruvic acid from tryptophan, lipolysis of Tween 20, Tween 40, Tween 60, Tween 80, and Tween 85, and proteolysis of gelatin and casein. Adsorption of bromothymol blue was assessed on nutrient agar supplemented with 0.004% (wt/vol) triphenyltetrazolium chloride and 0.0025% (wt/vol) bromothymol blue (NBTA medium) (1a). Pigmentation was determined by examining colonies on nutrient agar after incubation for 5 days. Antimicrobial activity was determined by the method of Akhurst (2), using *Micrococcus luteus* as the indicator organism. Cells from nutrient or Luria-Bertani (29) broth cultures were suspended in 0.25× Ringer's solution and examined by phase-contrast microscopy for the presence of inclusions. All tests were conducted in duplicate at 28°C.

Hemolysis was determined on blood agar (15 g of proteose peptone, 2.5 g of liver hydrolysate, 5 g of yeast extract, 5 g of NaCl, 12 g of agar, water to a volume of 1 liter, 10% sheep or horse blood) after 5 days of incubation at 28°C. Hemo-

TABLE 2. Levels of DNA relatedness of *Photorhabdus* strains

Source of unlabelled DNA	RBR (%) with ³² P-labelled DNA from strain:										
	Hb ^T	NC162	D1	Tetuan	ItH211	C1	Meg	X1Nach	NZH	Q614	1216-79
Hb ^T		61	67	67				48	45	47	
Hm	109	60	77	63	42	51	57	49	57	50	52
C8404	77		82			43	50		59	51	
C8406	77		64			37	59		48	62	
NC162	75		59		48	47	39		40	59	
D1	71	55		85		38	57		43	49	49
Tetuan	66		96		41	44	50		46	47	53
K81	62		85		44	54	53		44	54	
HP88	71					44	51		51	65	
K80	57		65			36	56		54	52	
HV16	59	63	49	45	38	29	41	40	30	36	45
HI	56		61		43	43	40		43	49	
ItH211	59	41	46			49	61		49	52	
C1	32		51	53			75	68	66	49	
Habana	57		43			93	88		70	55	
Meg	32		50			91		71	58	52	53
HW79	44		44			73	76		65	56	
HFR86	49					73	68		79	48	
HL81	52		48			60	78		75	56	
HF85	55		34			66	69		71	55	
X1Nach	48	43	48	50		65	73		51	55	54
X1Lit	63			48		58		102	61	45	
NZH	47		41			64	67	70			
T327	45		47			56	72	67	82	54	55
Q614	44		48			38	57		49		58
1216-79	44	33	48			50	60		56	50	
2407-88							50			55	103
2617-87	46		44			41			41	50	100
3105-77			41			29	55			55	107
3265-86			44	43						57	100

lysis was also determined on tryptic soy agar supplemented with 5% sheep blood (Oxoid) at 25°C.

Temperature tolerance was determined by suspending cells from 2- to 3-day nutrient agar cultures (28°C) in Luria-Bertani broth (29) and spotting 5-µl aliquots onto duplicate nutrient agar plates; for each strain one plate was incubated at 28°C and the other plate was incubated at 37°C. The plates were examined for growth daily for 5 days.

The phenotypic data were examined by performing a numerical analysis. The Jaccard coefficient (39) was used to calculate levels of similarity, and various clustering strategies (L&W flexible, unpaired group mean averages, ISS flexible) were applied. The whole data set, the whole data set minus the data for the major-phase characters, the whole data set minus the data for the major- and minor-phase characters, the phase I data set, and the phase II data set were analyzed. The data were also analyzed by using a strategy adopted for the genus *Xenorhabdus* (7) in which the data for both phases of each strain were combined and the strain was scored positive for a character if either phase was positive and negative only if both phases were negative.

Compatibility of *Heterorhabditis* and *Photorhabdus* strains. Various combinations of *Heterorhabditis* and *Photorhabdus* strains were grown in monoxenic cultures to assess their compatibility. For each test, the *Heterorhabditis* strain tested was cultured with its natural symbiont as a positive control and with *Photorhabdus* strains isolated from other *Heterorhabditis* strains.

Autoclaved pieces of polyether polyurethane sponge lightly coated with Bedding's medium (11) were placed on nutrient agar slants and inoculated with 1-ml portions of overnight cultures of *Photorhabdus* strains. Each sponge-containing slant was incubated at 28°C for 3 days to ensure complete coverage of the sponge and agar by the inoculated strain, which prevented growth of any *Photorhabdus* cells carried inside the infective juveniles. *Heterorhabditis* infective juveniles were surface sterilized by immersion in 0.1% thimerosal for 1 h and washed in sterile distilled water, and about 100 were added to each slant. The cultures were incubated at 28°C, and those cultures in which a new generation of infective juveniles was produced were subcultured by using the same procedure involving preinoculation of medium and surface sterilization of infective juveniles. Combinations were considered compatible when reproduction continued in the second subculture.

RESULTS

DNA relatedness. Levels of DNA relatedness were estimated for 172 pairs of isolates on the basis of the results of 286 hybridization reactions (Table 2). There was no significant ($P < 0.05$) difference from a 100% relative binding ratio (RBR) for phase I and phase II cultures of strains Hb^T, Meg, and K81 (106, 104, and 109%, respectively). There was also no significant ($P < 0.05$) difference in RBR for the reciprocal hybridizations performed with any of the 25 pairs tested or for hybridizations between any strain and the two phases of strains Hb^T, C1, Meg, K81, and T327.

Strains C1 and ATCC 29304, which were isolated from the same nematode strain in different laboratories in 1977 and 1983, had an estimated RBR of 101%. Several other pairs of strains derived from different nematode strains were found to exhibit 100% relatedness (e.g., strains Hb^T and Hm, strains X1Nach and X1Lit, and pairs of clinical isolates).

Phenotypic characterization. Both phases of all strains were positive for catalase activity and for assimilation of glucose, fructose, mannose, *N*-acetylglucosamine, fumarate, and L-glutamate. Both phases of all strains were negative for oxidase activity, nitrate reductase activity, the *o*-nitrophenyl-β-D-galactopyranoside-*p*-nitrophenyl-β-D-galactopyranoside test, arginine dehydrogenase activity, lysine decarboxylase activity, ornithine decarboxylase activity, H₂S production, and assimilation of erythritol, D-arabinose, L-arabinose, L-xylose, adonitol, methyl-D-xyloside, galactose, sorbose, rhamnose, dulcitol,

sorbitol, methyl-D-mannoside, methyl-D-glucoside, cellobiose, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-xylose, D-tagatose, D-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate, butyrate, isobutyrate, isovalerate, *N*-caproate, heptanoate, caprylate, pelargonate, caprate, malonate, malate, glutarate, adipate, pimelate, suberate, azelate, sebacate, glycolate, DL-lactate, DL-3-hydroxybutyrate, D-tartrate, meso-tartrate, levulinate, 2-ketoglutarate, citraconate, itaconate, mesaconate, phenylacetate, benzoate, 2-hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, D-mandelate, L-mandelate, phthalate, isophthalate, terephthalate, L-leucine, L-isoleucine, L-norleucine, L-valine, DL-norvaline, DL-2-aminobutyrate, L-methionine, L-phenylalanine, D-tryptophan, L-tryptophan, trigonelline, L-lysine, L-citrulline, betaine, creatine, β -alanine, DL-3-aminobutyrate, DL-4-aminobutyrate, DL-5-aminobutyrate, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, urea, acetamide, sarcosine, ethylamine, butylamine, amylamine, ethanolamine, benzylamine, diaminobutane, spermine, histamine, tryptamine, glycine, and DL-kynurenine.

The data for discriminatory phenotypic characters are shown in Table 3. All strains were highly proteolytic and lipolytic (for Tween 20), at least in phase I. Protoplasmic inclusions were produced by phase I organisms in all strains and to a lesser extent or not at all by phase II organisms. Bioluminescence was detected by eye in the clinical strains and in phase I cultures of all symbiotic strains except Q614, for which bioluminescence could not be detected even with a scintillation counter.

Phase I isolates of all strains adsorbed dyes from MacConkey agar and NBTa medium and exhibited antibiotic activity against *M. luteus*; phase II isolates were all negative for these characters. There were differences in proteolytic activity as determined with casein and gelatin for only 6 of the 53 isolates.

The unusual hemolytic reaction reported by Farmer et al. (20) was observed for both the clinical strains and some symbiotic strains on sheep blood agar at 25 and 28°C; this unusual reaction was more common at 25°C than at 28°C (Table 3). Only six isolates produced the unusual hemolytic reaction on horse blood agar at 28°C and none of the clinical strains exhibited this reaction under these conditions. In general, the unusual hemolytic reaction consisted of partial hemolysis around the colony, as demonstrated by greening associated with the production of biliverdin and annular clearing of the medium (total hemolysis?) at a distance of 10 mm or so from the colony. In a few isolates (Q614/1, HP88/2, and HV16/2), the annular clearing was overtaken after 3 or 4 days by total hemolysis that spread outward from the colony.

Numerical analysis of the phenotypic data from this study revealed that phase I variants of *Photorhabdus* isolates were generally more similar to each other than to the phase II variants of the same strains (Fig. 1), as previously demonstrated for *Xenorhabdus* isolates (7). Further examination revealed that antibiosis, dye binding, and phospholipase activity were the major phase-related characters in *Photorhabdus* and that these characters differentiated phase I variants from phase II variants in 23 or 24 of the 24 strains for which both phases were available. Differential pigmentation, bioluminescence, inclusion proteins, and partial hemolysis of sheep blood were also significant phase-related characters which differentiated the two phases of 15 to 19 of the 24 symbiotic strains. However, numerical analysis of the data after exclusion of these characters showed that there was still a slight tendency for isolates to cluster by phase prior to clustering as strains. Only 9 of the 65 discriminative characters (Table 3) did not differentiate the phases of any strain.

Numerical analysis of the phase I data set, the phase II data

set, and the combined data set did not produce any groups that correlated with either the DNA relatedness data or source (e.g., nematode host species).

Compatibility of *Heterorhabditis* and *Photorhabdus* strains. Testing combinations of various *Heterorhabditis* and *Photorhabdus* strains in vitro revealed some significant differences between strains (Table 4). There was no correlation between compatibility profile and DNA relatedness. Strains Hb^T and HI, which had an RBR of 56% (Table 2), had identical compatibility profiles, as did strains NZH and T327 (RBR, 82%), but strains Hb^T and D1 (RBR, 67 to 70%) had very different compatibility profiles.

DISCUSSION

This study showed that the strains of *P. luminescens* that are symbiotically associated with entomopathogenic nematodes can be distinguished from the clinical *P. luminescens* strains (20) by both phenotypic and DNA relatedness data. However, the clear functional differences in nematode-bacterium interactions among the symbiotic strains (Table 4) did not correlate with either phenotypic or DNA relatedness data. Only two groups, the symbionts and the clinical strains, could be identified by either phenotypic or DNA relatedness data. The hypothesis that there are five DNA relatedness groups in the genus *Photorhabdus* (20, 24) was disproved by the results of this study, which involved a larger number of *Photorhabdus* strains from wider ranges of host species and geographical sources than previous studies.

Phenotypic characterization. Farmer et al. (20) described an unusual hemolytic reaction that occurred in clinical strains when they were grown at 25°C, although not when they were grown at 37°C. This type of hemolysis, which is expressed as a thin band at some distance (ca. 10 mm) from each bacterial colony, generally with partial hemolysis immediately adjacent to the colony, was detected in some strains in the present study and was confirmed to be temperature sensitive. Farmer et al. (20) proposed that this reaction should be designated "*Xenorhabdus luminescens* hemolysis." However, as this type of hemolysis does not occur with the majority of *P. luminescens* strains and it has been proposed that *X. luminescens* should be transferred to the genus *Photorhabdus* (14), this term is not appropriate. We propose that the reaction should be designated "annular hemolysis" because it produces an annulus around each colony.

The analysis of the phenotypic data was complicated by the phase variation (1a, 13) and colonial heterogeneity (8, 19, 22, 23, 26) that occur in the symbiotic *Photorhabdus* strains and are correlated with biochemical characters often used in phenotypic taxonomy. It was further complicated by the absence of any phase I variants of the clinical strains. In the genus *Xenorhabdus* the phase problem was overcome by combining the data for the two phases and scoring a strain positive if either or both phases gave a positive response and negative only if both phases gave a negative response (7). However, this was not an option in this study because no phase I data could be obtained for the clinical strains, which did not produce as phase I variants. The taxonomy of the genus *Photorhabdus* had to be based on the results of a comparison of data that did not include data for the major phase-related phenotypic characters, and the resulting data set was too small to allow a meaningful numerical analysis. The use of only the extreme variants of each strain may have limited this study. A much larger phenotypic study performed with a range of variants for a suitably large number of strains of diverse origin may be required to establish a reliable taxonomy based on phenotypic data.

TABLE 3. Phenotypic characters that discriminate *Photorhabdus* isolates^a

[illegible]

TABLE 4. Incompatibility of some *Heterorhabditis* and *Photorhabdus* strains in in vitro culture

Strain	Compatibility with the following <i>Photorhabdus</i> strains:							
	Hb ^T	Hm	C1	HI	D1	NZH	T327	Q614
<i>Heterorhabditis bacteriophora</i> Hb	+	+	+	+	—	+	—	—
<i>Heterorhabditis bacteriophora</i> C1	+	+	+	+	—	+	+	—
<i>Heterorhabditis</i> sp. strain HI	+	+	+	+	—	+	—	—
<i>Heterorhabditis</i> sp. strain D1	—	—	—	—	+	—	—	—
<i>Heterorhabditis zealandica</i> NZH	—	—	+	—	—	+	+	+
<i>Heterorhabditis zealandica</i> T327	—	—	+	—	—	+	+	+
<i>Heterorhabditis</i> sp. strain Q614	—	—	—	—	—	+	+	+

symbiotic strains may arise from the level of variability within the symbiotic group. These findings do not invalidate the hypothesis that the clinical strains form a separate DNA relatedness group; they may reflect the diversity of the symbiotic group.

Although there appear to be some subsets within the symbiotic strain group, the degree of variability was very high and no subgroups could be defined. One apparent subset was made up of the Hb^T, Hm, C8404, C8406, NC162, D1, Tetuan, K81, HP88, K80, HV16, and HI strains (Table 2), which generally exhibited >60% relatedness, and another apparent subset was made up of strains C1, Habana, Meg, HW79, HFR86, HL81, HF85, X1Nach, X1Lit, NZH, and T327, which generally exhibited >65% relatedness. However, several strains exhibited about the same levels of relatedness to members of both subsets or higher levels of relatedness to members of the other subset than to some members of their own subset. The two symbiotic strains excluded from these subsets exhibited approximately equal levels of relatedness to members of both subsets. On the basis of these data it is not reasonable to subdivide the symbiotic strains.

Interpretation of the DNA relatedness data is complicated by the degree of experimental error involved. Although the estimates of DNA relatedness values for most reciprocal hybridizations were similar, some values varied more widely. However, analysis of the replicate data for reciprocal hybridizations revealed that there were no statistically significant differences for any pair. Estimates of levels of DNA relatedness in excess of 100% also indicate the extent of experimental error. The variations detected in this and other studies (24) show that care must be exercised in interpreting DNA relatedness data.

Conclusions. Despite the problems identified in this study, our phenotypic characterization and DNA relatedness data show that there are two distinct groups in the genus *Photorhabdus*. These groups are composed of the bacterial symbionts of entomogenous nematodes of the family *Heterorhabditidae* and of strains that have been isolated from human clinical specimens. The differences in the interactions of the symbiotic strains with various strains and species of nematodes (8, 25) and, to some extent, the DNA relatedness data suggest that the symbionts of nematodes are not a homogeneous group. However, in this study we did not identify phenotypic characters that differentiate organisms belonging to this group, even at the subspecies level. As the phenotypic differences of the symbiotic *P. luminescens* strains make it difficult to identify phenotypic characters that unambiguously differentiate the clinical group from the symbionts, it would be premature to define a new species for the clinical strains.

The challenge is to find the basis of the differences among the symbiotic *Photorhabdus* strains and to use that in constructing a valid taxonomy. There are a number of options that can

be identified at this time. One involves further investigation of the symbiotic specificity by gnotobiological experiments. This approach requires a more accurate definition of the *Heterorhabditis* species (see Table 1 for difficulties in naming nematode hosts). Cross-breeding (17) and molecular methods (27) are currently being used to provide this definition. However, this is a functional taxonomy and will not necessarily reflect phylogenetic relationships.

An attempt to determine the phylogeny of the genera *Photorhabdus* and *Xenorhabdus* by comparing the 16S rRNA gene sequences of strains led Rainey et al. to suggest that these two genera are closely related because the genomic differences between the type species of the two genera were apparently not significantly higher than the genomic differences found in *Xenorhabdus* species (36). However, these authors acknowledged that their analyses produced variable results and that the algorithms and reference strains used did not reliably depict the relationships between *Photorhabdus* and *Xenorhabdus* strains. They suggested that additional sequence data, especially for *P. luminescens*, will be required to improve the analyses.

Another approach to determining the phylogenetic relationships within the genera *Photorhabdus* and *Xenorhabdus* and between these genera is analyzing the origins of genes acquired to exploit their current niches. The phylogenetic differences between the nematode hosts (31) and significant differences in the molecular nature of some characteristics (e.g., intracellular crystal proteins) suggest that similarities within and between the genera *Photorhabdus* and *Xenorhabdus* result from convergent evolution. Many of the characteristics that are common to the two genera and are coordinately affected by phase variation may have been acquired to enable the organisms to exploit the symbiotic niche. The suggestion (21) that molecular analysis of genes such as the protease, lipase, and intracellular crystal protein genes, etc. might be useful for distinguishing between *Photorhabdus* strains is well worth pursuing.

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